

Molecular discrimination and antifungal susceptibility profile of cryptic *Candida albicans* complex species isolated from patients in Iran

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ABSTRACT

Background and Objectives: *Candida albicans* complex species are well known as the main cause of candidiasis, particularly among susceptible individuals. In this study, we report the genetic diversity of *Candida* spp. and the antifungal susceptibility pattern of the cryptic *C. albicans* complex isolates in Kerman, Iran.

Materials and Methods: A total of 112 yeast isolates were obtained from different clinical samples, and molecular identification was performed. All *C. albicans* complex isolates were tested for susceptibility of them to amphotericin B, fluconazole, and itraconazole.

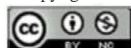
Results: The majority of clinical isolates were *C. albicans* complex (n=48) followed by *C. glabrata* complex (n=34), *C. parapsilosis* complex (n=21), and *C. krusei* (n=9). Among *C. albicans* complex, 45 isolates were *C. albicans* (94%), 2 isolates were *C. dubliniensis* (4%), and 1 isolate was *C. africana* (2%). Amphotericin B was the most active antifungal, whereas 8.9% and 6.7% of the isolates were resistant to fluconazole and itraconazole, respectively.

Conclusion: Regarding the high incidence of *Candida* infections particularly in susceptible populations and the emergence of an infrequent yeast species with elevated MICs, which is indistinguishable with conventional methods, developing accurate molecular methods for laboratory diagnosis should be considered in the clinical setting.

Keywords: *Candida albicans*; Candidiasis; Polymerase chain reaction; Amphotericin B; Itraconazole

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INTRODUCTION

Candida species are well known as normal flora which colonize on the mucosal membrane and the skin of healthy individuals (1). In addition, these opportunistic yeasts are responsible for a broad spectrum of diseases, particularly in immunocompromised patients with an endogenous or exogenous source of infections (2). Candidiasis also is a nosocomial infection that increases in length of hospital stay and even causes death if left untreated (3). According to CDC's surveillance data the mortality rate of invasive candidiasis among people with candidemia is approximately 19-24% (4).

Despite over 350 subspecies of *Candida* identified to date, only five species account for more than 90% of infections including *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei* (5). Some of these agents are "cryptic species," which currently morphological as well as biochemical methods unable to precisely distinguish these *Candida* species (6). To fulfill this gap molecular methods are increasingly developed for the accurate identification of *Candida* species (6, 7).

C. albicans is now considered a cryptic complex species, including *C. albicans*, *C. africana*, and *C. dubliniensis* (8, 9). Nowadays, with the hyphal cell wall protein 1 (*HWPI*) gene as a particular target, differentiation of the *C. albicans* complex species has been achieved (10). However, due to homozygous and heterozygous strains of *C. albicans* in the *HWPI* locus, two profiles pattern have been observed by PCR amplification (11). Moreover, timely and precise differentiation among this complex is substantial since these complex species may vary in pathogenicity and anatomic prevalence (12, 13). Besides, regarding different antifungal susceptibility patterns of *C. albicans* complex species, choosing the proper treatment is critical. Recently, emerged resistance to azole antifungal agents which are commonly used to treat *Candida* infections, have been become a clinical concern (14).

As *C. albicans* complex species represents an epidemiological concern worldwide that virulence and antifungal susceptibility pattern often vary among them; the challenge lies in species or interspecies discrimination, which misidentification of members of the *C. albicans* complex might lead to overestimation of *C. albicans* burden. Consequently, the main goal of this study was to differentiate all these medi-

cally important *Candida* species using PCR - RFLP and *HWPI* gene as molecular methods. In addition, we aimed to investigate the *in vitro* antifungal susceptibility of three antifungal drugs against *C. albicans* complex species obtained from patients in Kerman, Iran.

MATERIALS AND METHODS

Informed consent and ethics statement. A written informed consent form was completed for each patient. All clinical and demographic data were kept confidential. Also, parents/guardians provided written informed consent "on behalf "of all the children. All the patient-related data in this study were processed anonymously and approved by the ethics committee of Kerman University of Medical Sciences (Ethics approval code: IR.KMU.REC.1398.710).

Clinical samples and conventional phenotypic identification. A total of 112 fungal cultures were obtained from the patient referred to the reference mycology laboratory of Kerman University of Medical Sciences from April 2020 to May 2021. All positive yeast cultures were included in the study. Isolates were from bronchoalveolar lavage (BAL) (n = 43), sputum (n = 25), oral wounds (n = 21), nail (n = 13), skin (n = 9) and peritoneal fluid (n =1). All clinical specimens initially were tested by direct microscopic examination with KOH 20% and subcultured on Sabouraud dextrose agar (Merck, Germany) plates containing 0.5 µg/ml chloramphenicol (Merck, Germany). The cultures were incubated for 24 to 48 h at 30°C before testing. All *Candida* isolates were initially identified by subculturing on the chromogenic medium CHROMagar *Candida* (CHROMagar, India, Cat no: 212961), at 35°C for 48 h, to ensure isolation of pure colonies.

DNA isolation. DNA was extracted using the previously described Phenol-Chloroform-glass beads protocol with some modification (15). The quantity and purity of the DNA were calculated using 260/280 and 260/230 ratios with a NanoDrop Spectrophotometer (ND 1000), and the integrity was checked with 1.5% gel agarose electrophoresis.

Molecular identification: PCR-RFLP. *Candida* species were identified by the widely used method

based on PCR-RFLP. The ribosomal region comprising ITS and the adjacent region was directed to be amplified using the universal primer pairs ITS1 (5-TCCGTAGGTGAACCTGCGG-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3). The PCR was carried out in 25 μ l reaction volumes and included 8 μ l of 2 \times PCR master mix (Ampliqon, Co, Denmark), 2 μ l DNA as the template, 1 μ l of forward and reverse primer at a concentration of 10 μ M, and 13 μ L of distilled water. DNA was amplified according to the following program: initial denaturation 95°C for 5 min, 30 cycles of denaturation 95°C for 30 s, annealing 60°C for 60 s, and extension 72°C for 120 s, and a final extension at 72°C for 10 min. PCR products were visualized in 1.5% agarose gel electrophoresis in TBE 0.5 \times buffer with ethidium bromide. After confirming the presence of desired ribosomal region, 5 μ l of the PCR product were treated with the *MspI* restriction enzyme at 37°C for 2 h with 2 μ l of specific buffer, 0.5 μ l of the restriction enzyme (10 U/ μ l) (Thermo Scientific), and 2.5 μ l of distilled water to a final volume of 10 μ l. The digested fragments were separated through 2% agarose gel electrophoresis and visualized in a UV-transilluminators System and compared with the relative size of the reference fragment profiles (15).

PCR- HWPI. Cryptic species of *C. albicans* were investigated based on PCR amplification of the size polymorphism of *HWPI* gene using primers pairs CR-f (GCT ACC ACT TCA GAA TCA TCA TC) and CR-r (GCA CCT TCA GTC GTA GAG ACG) (10). The PCR was carried out in 20 μ l reaction volumes and included 7 μ l of 2 \times PCR master mix (Ampliqon, Co, Denmark), 2 μ l DNA as the template, 2 μ l of forward and reverse primer at a concentration of 10 μ M, and 9 μ l of distilled water. The thermocycler program was: 95°C for 5 min, 35 cycles of 95°C for 45 s, 58°C for 40 s, and 72°C for 55 s, and a final extension at 72°C for 10 min. PCR products were visualized in 1.5% agarose gel electrophoresis in TBE 0.5 \times buffer with ethidium bromide using a transilluminator. The reference PCR product sizes of *C. africana* is 700 bp, homozygous *C. albicans* strains: single band at ~941 pb, *C. albicans* strains heterozygous strains: two bands at 800 and 941 bp, and *C. dubliniensis*: 569 bp.

Antifungal susceptibility testing. According to the Clinical and Laboratory Standards Institute (CLSI) M27-A3 guideline, minimum inhibitory concentration (MICs), for all obtained *C. albicans* com-

plex isolates were determined. Three antifungal drugs were used in this study including amphotericin B, fluconazole, and itraconazole (Sigma-Aldrich, USA). The final concentrations of drugs were ranged from 0.031 to 16 μ g/ml for amphotericin B and itraconazole, and 0.125 to 64 μ g/ml for fluconazole. All identified *C. albicans* complex isolates were sub-cultured on Sabouraud Dextrose Agar (SDA Difco) plates at 35°C for 24 h and then standardized yeast inoculum of 0.5-2.5 $\times 10^3$ cells/ml in RPMI 1640 medium was added to each well of 96-well microplate. The microdilution plate was incubated at 35°C for 24 h and 48 h, and then MICs endpoint were determined as the lowest concentration by comparison to the drug free control well. According to the CLSI document, MIC breakpoints were interpreted for fluconazole (S: susceptible, ≤ 2 ; SDD: susceptible dose-dependent = 4; R: resistant ≥ 8 μ g/ml), itraconazole (S ≤ 0.125 ; SDD = 0.25–0.5; R ≥ 1 μ g/ml), and amphotericin B (S ≤ 1 ; R ≥ 2 μ g/ml). *C. parapsilosis* (ATCC 22019), and *C. krusei* (ATCC 6258) were used as quality control strains in all experiments. All *in vitro* susceptibility tests were performed in duplicate.

RESULTS

Phenotypic and molecular identification of isolates. A total of 112 clinical isolates of *Candida* from a variety of specimens were included in this study. All clinical yeast isolates were identified as *Candida* species based on conventional as well as molecular assays. In the current study, it was observed that chromogenic medium can differentiate between some *Candida* species. As shown in Fig. 1 *Candida* species

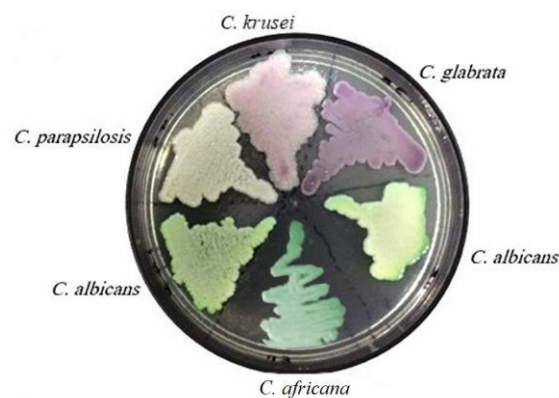


Fig. 1. Appearance of *Candida* species after 48 h of incubation at 35°C on CHROMagar

could be distinguished on the basis of its appearance on CHROM agar, in accordance with the manufacturer's recommendations. Totally, 48 out of 112 isolates presented a green colony color on the chromogenic medium, as *C. albicans* complex species. Colony morphology of *C. africana* reveals deeper turquoise-green than *C. albicans*.

An overview of obtained results from the distribution of various species based on the isolation source is summarized in Table 1. According to the results of the RFLP-PCR method, the majority of clinical isolates were identified as *C. albicans* complex (n=48) followed by *C. glabrata* complex (n=34), *C. parapsilosis* complex (n=21), and *C. krusei* (n=9). The patterns of ITS PCR-RFLP for *Candida* isolates after digestion with restriction enzyme *MspI*, are shown in Fig. 2.

Molecular identification of *C. albicans* cryptic species using *HWPI* gene amplification confirmed the genetic variability of these strains. *C. albicans* isolates

(n=45), were either homozygous with a single band at ~941 bp (27 strains) or heterozygous with two bands at 800 and 941 bp (18 strains). Only one strain showed a single band of ~ 569 bp, and two strains showed a single band of ~ 700 bp which correspond to *C. dubliniensis* and *C. africana*, respectively (Fig. 3).

Antifungal susceptibility profile. Table 2 shows the results of the MIC range, MIC90, and the geometric mean of three tested antifungal agents. Most of the *C. albicans* complex isolates (41 isolates; 85.41%) were susceptible to all of the antifungal agents tested. Amphotericin B was the most active antifungal, against all the tested *C. albicans* strains (100%) which showed a susceptible profile, whereas 8.9% and 6.7% of the isolates were resistant to fluconazole and itraconazole, respectively. Besides, 35 isolates (77.8%), and 6 isolates (13.3%) of *C. albicans* strains were susceptible and susceptible-dose dependent to fluconazole, re-

Table 1. Distribution of clinical isolates of *Candida* species based on the site of infection.

<i>Candida</i> spp.	BAL	Sputum	Oral wounds	Nail	Skin	Peritoneal fluid	Total
<i>C. albicans</i>	18	12	7	7	1	-	45
<i>C. dubliniensis</i>	-	-	2	-	-	-	2
<i>C. africana</i>	-	-	1	-	-	-	1
<i>C. glabrata</i> complex	15	5	6	4	4	-	34
<i>C. parapsilosis</i> complex	6	5	3	2	4	1	21
<i>C. krusei</i>	4	3	2	-	-	-	9
Total	43	25	21	13	9	1	112

BAL: Bronchoalveolar lavage

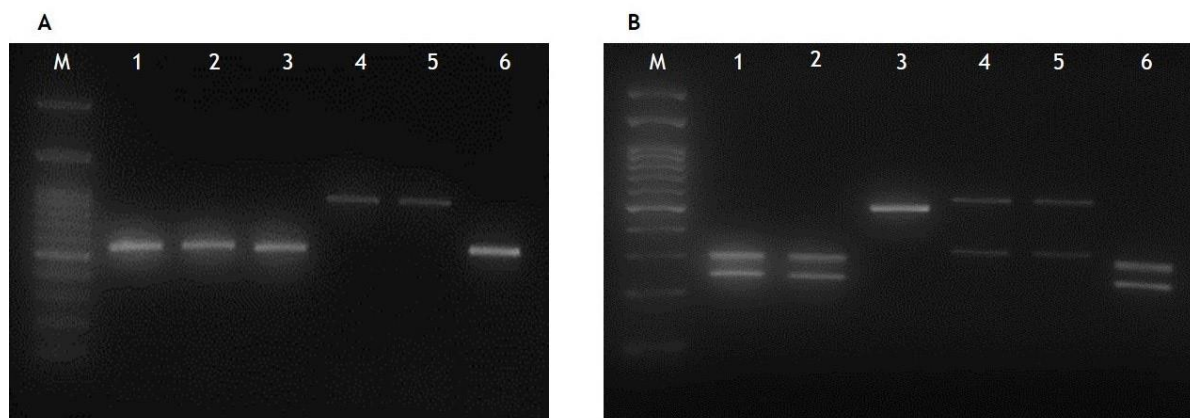


Fig. 2. A) Lanes: M, 100 bp DNA size marker. Lanes 1-6: Agarose gel electrophoresis of ITS-PCR products of *Candida* species before digestion. B) Agarose gel electrophoresis of ITS-PCR products of *Candida* species after digestion with *MspI*; lanes 1, 2, and 6: *C. albicans* complex species, lane 3: *C. parapsilosis* complex species, lanes 4 and 5: *C. glabrata* complex species. Lane M: a 100-bp DNA size marker.

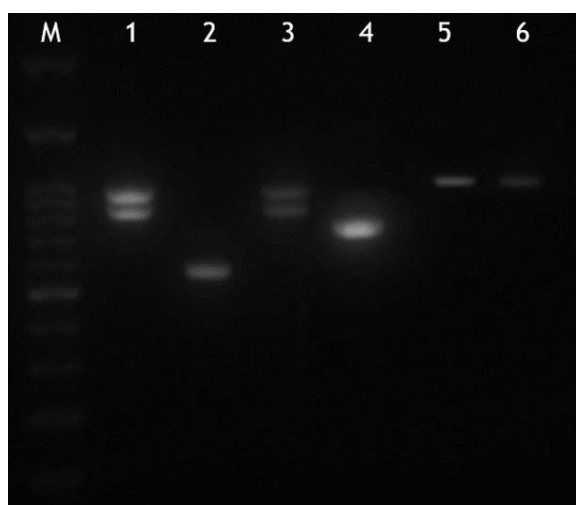


Fig. 3. Electrophoretic profile of Hwp1 amplification. Lanes: M, 100 bp DNA size marker. Lanes 1 and 3: Heterozygous strains of *C. albicans* isolates (800 and 941 bp), Lane 2: *C. dubliniensis* (569 bp), Lane 4: *C. africana* (700 bp), Lanes 5 and 6: Homozygous strains of *C. albicans* isolates (941 bp).

spectively. On the contrary, 23 isolates (51.1%) were susceptible, and 19 isolates (43%) were susceptible-dose dependent to itraconazole. Two *C. dubliniensis* and one *C. africana* strains were susceptible to all the tested antifungals.

DISCUSSION

Recently non-*albicans Candida* (NAC) spp., such as *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and their atypical species have become an emerging cause of candidiasis (16, 17). Nevertheless, *C. albicans* complex is well known as the predominant cause of candidiasis in the general population (6). In this study, we report the genetic diversity, as well as antifungal susceptibility pattern of cryptic *C. albicans* complex isolates from clinical specimens in Iran.

Patterns of ITS PCR-RFLP showed a high frequency of *C. albicans* complex (42.85%) followed by *C. glabrata* complex (30.35%), *C. parapsilosis* complex (18.75%), and *C. krusei* (8.03%) isolates. Several studies reported similar findings which indicated *C. albicans* complex species are among the most common cause of infections around the world and increasing morbidity cases turned it into a serious public health problem (18). On the other hand, species within the *C. albicans* complex are more often involved in human fungal infections. In the current study, by amplification of the *HWPI* gene initial identification of *C. albicans* complex species were further identified as *C. albicans* (94%), *C. dubliniensis* (4%), and *C. africana* (2%). Moreover, as mentioned in the results *C. albicans* isolates were either homozygous (27 strains) with a single band at ~941 pb, or heterozygous with two bands at 800 and 941 bp (18 strains) at the *HWPI* locus. Although data on the homozygous and heterozygous strains of *C. albicans* isolates are exceedingly poor, some reports indicated that there are no differences in virulence factors and pathogenesis of these strains (19). We recommended surveying this heterogeneity in the physiological and biochemical aspects of this pathogenic yeast in future studies.

We also assessed the *in vitro* antifungal activity of three antimycotic agents against a collection of 48 *C. albicans* complex isolates. According to *in vitro* antifungal susceptibility results, amphotericin B had excellent activity against all *C. albicans* strains and 100% of strains showed a susceptible profile, whereas 8.9% and 6.7% of the isolates were resistant to fluconazole and itraconazole, respectively. These results are consistent with the results of previous studies which shown *C. albicans* isolates are normally susceptible to amphotericin B. Likewise, Sharifinia et al. reported that 16.1% and 21.9% of the *C. albicans* isolates were resistant to fluconazole and

Table 2. *In vitro* susceptibilities of three antifungal drugs against 48 clinical isolates of *C. albicans* complex.

<i>Candida</i> species	Antifungal drugs (µg/ml)								
	AMB			FLU			ITC		
	MIC ₉₀	range	GM	MIC ₉₀	range	GM	MIC ₉₀	range	GM
<i>C. albicans</i> (n=45)	0.125	0.016-0.5	0.0709	4	0.5->32	1.4697	0.25	0.031-2	0.1527
<i>C. dubliniensis</i> (n=2)	0.031	-	-	0.5	-	-	0.063	-	-
<i>C. africana</i> (n=1)	0.016	-	-	0.5	-	-	0.031	-	-

AMB: amphotericin B, FLU: fluconazole, ITC: itraconazole, MIC: minimum inhibitory concentration, GM: geometric mean.

itraconazole, respectively (20).

Furthermore, although *C. africana* mostly recovered from vaginal samples worldwide, some studies have been reported isolation from different clinical samples, which suggests this pathogen also could be associated with a broader clinical spectrum (21, 22). Besides, some recent studies have shown that *C. africana* strains exhibited to be resistant to some antifungal agents (23, 24). In the current study, only one *C. africana* isolate was obtained as a cause of oropharyngeal candidiasis in MML cancer patient. Our results of the *in vitro* susceptibility testing for *C. africana* as well as *C. dubliniensis* isolates showed low MIC values for all the antifungal drugs tested. It is quite remarkable that using different classes of antifungal agents is essential for the reliable evaluation of the *in vitro* susceptibility profiles of these *Candida* species. This aspect requires further epidemiological studies, to determine the real prevalence and understand the extent of antifungal resistance of these pathogenic yeasts.

In conclusion, regarding the high incidence of *Candida* infections particularly in susceptible populations and the emergence of an infrequent yeast species with elevated MICs, which are indistinguishable with conventional methods, developing accurate identification molecular methods should be considered in the clinical setting.

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